Cynaropicrin Shows Antitumor Progression Potential in Colorectal Cancer through Mediation of the LIFR/STATs Axis

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Abstract

Background: Colorectal cancer (CRC) is the second deadliest malignant disease in the world, with a large number of new cases being reported every year. The leukemia inhibitory factor receptor/signal transducers and activators of transcriptions (LIFR/STATs) signaling axis plays an important role in the molecular biology of CRC. The inactivation of STATs is a promising anticancer strategy. Cynaropicrin is a sesquiterpene lactone with a variety of pharmacological functions, which may be used as a potential targeted cancer drug for the prevention or treatment of human CRC.

Methods: Cell function tests were performed to observe the inhibitory effect of Cynaropicrin on human CRC cells (RKO, HCT116 and DLD1). Expression levels of LIFR, P-STAT3, P-STAT4 and apoptotic proteins were detected by Western blotting. Immunoprecipitation confirmed the presence of LIFR/STAT3/STAT4 complex. Cell immunofluorescence assay was used to observe the subcellular localization of STAT3 and STAT4. In vivo efficacy of Cynaropicrin was evaluated by a xenotransplantation model in nude mice.

Results: Cynaropicrin significantly reduced the survival ability of human CRC cells and promoted apoptosis in a dose-dependent manner. Western blotting results suggested that the antitumor effects of cynaropicrin might be mediated by inhibition of the LIFR/STATs axis. Cynaropicrin reduced the formation of STAT3/STAT4 heterodimers and blocked their entry into the nucleus. Cynaropicrin also suppressed tumor growth in the HCT116 xenograft model.

Conclusion: The above experimental results showed that cynaropicrin exerted a strong inhibitory effect on CRC *in vitro* and *in vivo*. Our study concluded that cynaropicrin has potential application prospects in the field of anti-CRC therapy.

Introduction

Colorectal cancer (CRC), one of the most prevalent malignant diseases, ranks second among all cancers in terms of mortality and third in terms of incidence worldwide (1). There are projected to be 147,950 individuals newly diagnosed with CRC and an estimated 53,200 CRC deaths in the United States in 2020 (2). In the Chinese population, CRC was one of the most common causes of cancer-related deaths in males (8.0%) and females (9.8%) (3). Therefore, in addition to surgery, there is an urgent need to identify promising molecular drugs with high efficacy and low toxicity.

Leukemia inhibitory factor (LIF) is a member of the interleukin-6 (IL-6) family and has the most multi-potent action. LIF binds to leukemia inhibitory factor receptor (LIFR) with high affinity (4), activating the Janus Kinase (JAK) family of tyrosine kinases, particularly JAK1 (5). JAK1 activates a series of tyrosine phosphorylation events and stimulates three signaling pathways, including JAK/signal transducers and activators of transcription (STATs) (6). Signal STAT3, the only STAT family member whose genetic deletion results in embryonic lethality (7), has been estimated to be abnormally activated in more than 70% of human cancers (8). Persistent STAT3 activation is involved in tumorigenesis, proliferation, anti-apoptosis, and metastasis (9). STAT4, also a member of the STAT family, is a key mediator of the pro-

inflammatory immune responses. High expression of STAT4 has been shown to be a positive prognostic factor in liver cancer, breast cancer, and ovarian cancer (10), whereas its overexpression in CRC is positively correlated with the depth of tumor invasion (11). STAT3 is considered to be the most important signal transducer of LIF stimulation. It has been shown that LIF cytokine stimulation induces LIFR to recruit the STAT protein (12). Further, the STAT protein is activated to form a signal-enhanced dimer that enters the nucleus and upregulates the transcription of the corresponding cytokine response genes (13).

Cynaropicrin, a sesquiterpene lactone (STL), is a major biologically significant class of secondary metabolites in the artichoke (14). Cynaropicrin has been shown to possess various pharmacologic properties, such as anti-hepatitis C virus (15), anti-parasitic (16), anti-photo aging (17), anti-inflammatory (18), and anti-tumor properties. In human leukemia and prostate cancer cells, cynaropicrin is able to induce glutathione depletion and result in S-glutathionylation of STAT3, leading to down-regulation of STAT3-dependent gene expression and chemosensitization of tumor cells to chemotherapy (19). Cynaropicrin impinges on the thioredoxin (Trx) system and leads to Trx oxidation and reactive oxygen species (ROS) accumulation in cells, thereby inducing apoptosis of Hela cells (20). Cynaropicrin may serve as a potential cancer targeted drug for prevention or treatment of human cancers.

Inactivation of STAT3 is a promising anticancer strategy, but an STAT3 inhibitor has not yet been approved for the market. As a potential natural product targeting the STAT3-related signaling pathway, the application of cynaropicrin in CRC may be worth exploring. Therefore, we assessed the inhibitory effect of cynaropicrin on CRC tumors at the cellular and animal levels. In this study, we further explored the specific anti-cancer mechanism of cynaropicrin to determine if it has potential application prospects in the field of antineoplastic therapy.

Experimental

Cell culture and reagents

Human colon cancer cell lines (HCT116, RKO and DLD-1) were obtained from the cell resources center of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). HCT116 cells were grown in McCoy's 5A medium (Gibco, New York, USA) and DLD-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, USA). RKO cells were cultured in RPMI-1640 media (Thermo Fisher Scientific, Waltham, USA). The above-mentioned basic culture media were supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, USA) and 1% of Penicillin-Streptomycin (10,000 U/mL) (Thermo Fisher Scientific, Waltham, USA) and incubated at 37°C with 5% CO₂. Cynaropicrin was purchased from Baoji Herbest Bio-Tech Co. Ltd. (CAS#: 35730-78-0). Recombinant human IL-6 was purchased from Bio-Techne China Co. Ltd. (206-IL-010).

MTT assay

Cells were seeded into wells of a 96-well plate (3×10^3 cells/well) with 100 μ L of the corresponding medium and allowed to attach overnight. Cynaropicrin was dissolved in DMSO to a certain concentration using gradient dilution. After being incubated with Cynaropicrin for 48 h, the cells were treated with 25 μ L/well MTT solution (5 mg/ mL) for 4 h at 37 °C. The formazan crystals were dissolved in 150 μ L DMSO and the optical density (OD) was measured using a Microplate Reader at 490 nm. Half-maximal inhibitory concentration (IC₅₀) values were determined by GraphPad Prism 7.0.

Colony formation assay

The cells were seeded into a 6-well plate (800 cells/well) and incubated overnight. After treatment with drugs or DMSO for 2-6 h, the culture medium was replaced with fresh medium to keep the cells growing for one week. Colonies were fixed with 4% methanol for 15 min at room temperature and then stained with 1% crystal violet for 10 minutes at room temperature. After staining, the plates were washed with phosphate-buffered saline (PBS) and dried.

Assessment of cell apoptosis by flow cytometry

Apoptosis was detected by FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™, USA). In brief, cells inoculated in a 6-well plate were collected after being treated with DMSO or drugs for 24 h. Then they were resuspended in 500 μ L binding buffer according to the instructions of the apoptosis kit. The treated cells (as described above) were successively incubated with fluorescein-labeled Annexin V and propidium iodide (PI). Apoptosis assessment was performed by FACSCalibur (BD Biosciences, MD, USA). Data were analyzed using Flowjo software.

Cell migration assay

Cell migration assays were performed using a transwell filter (BD Biosciences, USA) according to the manufacturer's instructions. Cells were seeded in the upper chamber containing a non-coated membrane. Culture medium containing 10% FBS was added to the lower chambers, and the cells were plated in the upper chamber with FBS-free medium. Both media were treated with DMSO or drugs. After 48 h, the cells were fixed with 4% paraformaldehyde and non-migrated cells were removed from the upper surface of the filter. The cells on the lower surface of the membrane were stained with 0.1% crystal violet for 10 min. The number of migrated cells was visualized and counted under an optical microscope.

Immunoprecipitation and Western blotting

Cells were lysed in protein lysis buffer and centrifuged to obtain the supernatant. Lysates were incubated overnight with the appropriate antibody and agarose beads to isolate the target proteins. Proteins were separated by 10% or 12% SDS-PAGE and then transferred onto a PVDF membrane. The blots were

blocked for 2 h with fresh 5% non-fat milk at room temperature, followed by incubation with specific primary antibodies overnight at 4°C. After washing, membranes were incubated with the relevant secondary antibodies. Antibody staining was visualized using Omni-ECL™ Femto Light Chemiluminescence Kit (EpiZyme, Shanghai, China). Then, the images were analyzed by the Image J computer software. The following primary antibodies were commercially obtained: anti-GAPDH (AB-P-R 001, GoodHere Technology), anti-STAT3 (phospho Y705) (ab76315, Abcam), Stat3 mAb (#12640, Cell Signalling Technology), Phospho-Stat4 (Tyr693) mAb (#4134, Cell Signalling Technology), Stat4 mAb (#2653, Cell Signalling Technology), anti-LIFR (sc-515337, Santa Cruz), anti-LIF (sc-515931, Santa Cruz), anti-Bax (ab32503, Abcam), anti-Bcl-2 (sc-56015, Santa Cruz), and Anti-Cleaved PARP1 (ab32064, Abcam).

Immunofluorescence

The cells seeded in glass bottom cell culture dish treated with Cynaropicrin and/or IL-6 were fixed with 4% formaldehyde and then permeabilized in 0.1% Triton X-100. The fixed colorectal cancer cells were blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Blocked cells were incubated with the specific primary antibody of STAT3 (1:500 in 1% BSA) or STAT4 (1:1600 in 1% BSA) overnight at 4°C. After rewashing in PBS, the cells were allowed to react with Goat Anti-Rabbit IgG (Alexa Fluor® 488) (ab150077, Abcam) (1:700 in 1% BSA) for 1 h in the dark and counterstained with DAPI for 10 min. The images of STAT3/STAT4 and DAPI stained cells were observed under a Leica SP5 confocal microscope.

Xenograft models

All animal experiments were conducted using protocols approved by The Wenzhou Medical University Animal Policy and Welfare Committee. Hct116 cells mixed with an equal volume of PBS and matrigel were implanted in the hind flank of mice (nude mice, female, 5–6 weeks old). Upon attaining an appropriate tumor volume (approximately one week post-implantation), the mice were randomized into 4 groups and intraperitoneally injected with Napabucasin or Cynaropicrin. Tumor volume was measured as $V = (L \times W \times W) / 2$ (L: length and W: width). Animals were sacrificed at the end of study. The tumors, heart, liver, kidney and lung were removed and preserved in 4% paraformaldehyde for further use (histological and protein expression analyses).

Statistical analysis

The experimental results were expressed as the mean $\pm$ SDs of three parallel experiments. The differences in data among groups were analyzed by unpaired two-tailed Student's t-tests in GraphPad Prism 7. *P*-values less than 0.05 were considered statistically significant.

Results

Cynaropicrin affected the cell viability of CRC

To investigate the inhibitory effect of cynaropicrin-inhibited proliferation in human CRC cell lines, cell viability was evaluated (Figure 1A). The results of MTT assay showed that cynaropicrin exhibited promising growth inhibition in HCT116, RKO, and DLD-1 cells in a dose-dependent manner. The corresponding IC₅₀ values were 4.45 μ M, 3.89 μ M and 8.88 μ M, respectively (Figure 1B). Furthermore, the colony formation assay showed that cynaropicrin can significantly suppress the colony formation ability of these three cell lines as shown in Figure 1C. Together, these data suggested that cynaropicrin can effectively inhibit the growth and proliferation of human CRC cells.

Induction of the apoptosis effect of cynaropicrin in CRC cells

To evaluate whether cynaropicrin can induce cell apoptosis, three cell lines were treated with cynaropicrin at three different concentrations for 24 h, stained with Annexin V FITC and PI, and the percentage of apoptotic cells was detected by flow cytometry. Remarkably, cynaropicrin significantly induced CRC cells apoptosis (Figure 2A). Similar results were provided by Hoechst 33258 staining, which further confirmed the above observations. Cells treated with cynaropicrin displayed strong blue fluorescence and demonstrated significant apoptotic patterns (Figure 2B). Here, we also found changes in the expression levels of Cleaved PARP1, BCL-2, and BAX, which are associated with the process of apoptosis (Figure 2C).

Cynaropicrin-induced loss of migration potential in CRC cells

To determine whether treatment with cynaropicrin was associated with tumor cell migration, changes in migration potential were measured using transwell assays. As shown in Figure 3, compared with the control group cynaropicrin significantly suppressed the cell migration capability. The number of cells passing through the transwell filtration membrane was significantly reduced after treatment with gradually increasing concentrations of cynaropicrin. Therefore, we concluded that cynaropicrin inhibited migration of the CRC cell lines in a concentration-dependent manner.

Cynaropicrin blocked the activation of STAT3 via inhibiting LIFR expression

In consideration of the important role played by the STAT3 pathway in CRC, we attempted to determine whether cynaropicrin can inhibit the phosphorylation and activation of STAT3 in CRC cells. Via detecting the protein expression level by Western blotting, it was found that treatment with cynaropicrin obviously controlled the level of phosphorylate-STAT3 in HCT116, RKO, and DLD-1 cells in a time- and dose-

dependent manner (Figure 4A-4B). These findings proved that cynaropicrin induced CRC cell death mainly through the STAT3 pathway. To further investigate the mechanisms of cynaropicrin-induced cell apoptosis, we assessed subcellular localization of the STAT3 protein. As shown in Figure 4C, cynaropicrin significantly inhibited IL6-induced STAT3 nucleation. The separation of the nucleoprotein and the plasma protein also verified that cynaropicrin blocked STAT3 translocation into the nucleus (Figure 4D). The above results strongly indicated that cynaropicrin inhibited STAT3 phosphorylation and its translocation into the nucleus.

Previous studies have demonstrated a strong role of STAT3 in LIF-signaling among several cell types (21). LIFR and GP130 tyrosine residues provide SH2 domains of the STAT proteins with specific docking sites, resulting in subsequent STAT phosphorylation (12). We verified the effect of cynaropicrin on the STAT3 upstream signaling pathway by demonstrating that the binding capacity of LIFR to STAT3 was inhibited after cynaropicrin treatment through protein immunoprecipitation (Figure 5A). Western blotting was used to detect the LIFR protein expression levels. The results are shown in Figures 4A and 4B. The inhibition of LIFR protein expression by cynaropicrin treatment was enhanced in a dose- and time-dependent manner. Moreover, LIFR expression was reduced prior to STAT3 phosphorylation (Figure 4B). In conclusion, we suggest that cynaropicrin inhibited LIFR expression, thereby blocking the downstream activation of the STAT protein.

Cynaropicrin stimulation inhibited the formation of STAT3-STAT4 heterodimers

After activation by LIF/LIFR, STAT3 can not only be activated to form a homodimer, but also to form a heterodimer with STAT4 (22). We assessed the influence of cynaropicrin on the formation of STAT3 dimer, and the results of Co-Immunoprecipitation (Co-IP) using antibodies directed against STAT3 and STAT4 are shown in Figures 5A and 5B. The changes in protein bands strongly suggested that the formation of the STAT3-STAT4 heterodimer was inhibited by cynaropicrin. After drug treatment, the binding of STAT3 to LIFR was also negative (Figure 5A). Cellular immunofluorescence assay also demonstrated that STAT4 failed to translocate into the nucleus after cynaropicrin treatment (Figure 5C), which was consistent with the finding that STAT3 translocation into the nucleus was blocked. Therefore, it can be basically concluded that cynaropicrin inhibited LIFR recruitment of STAT3/4 and further reduced STAT3-4 heterodimer formation.

Cynaropicrin inhibited the growth of CRC xenograft models

BALB/c nude mice were inoculated subcutaneously with HCT116 human CRC cells and used as xenograft models to evaluate whether cynaropicrin can inhibit the growth of cancer cells *in vivo*. The STAT3 inhibitor Napabucasin was used as the positive control. Our results showed that intraperitoneal administration of cynaropicrin at doses of 2.5 mg/kg and 5 mg/kg resulted in decreased tumor volume

and weight compared to the vehicle group and Napabucasin (10 mg/kg) group (Figure 6A-C). We found that LIFR and STAT3 phosphorylation was mechanistically inhibited in the treatment groups. Drug treatment also markedly increased apoptosis as indicated by the expression levels of Bax and Bcl-2 (Figure 6D). Moreover, no significant loss of body weight occurred in any of the treatment groups (Figure 6E). We also evaluated the toxicity of cynaropicrin by hematoxylin eosin (H&E) staining analysis in mouse heart, liver, lung and kidney. No obvious cellular inflammatory, edema or necrosis was observed, demonstrating an excellent safety profile (Figure 6F). These results showed that cynaropicrin has potent antitumor activity against the growth of implanted CRC with minimal toxicity in the animal.

Discussion

STAT3 has important biological significance and is a potential therapeutic target for CRC (9, 23). Clinical studies in 724 patients with stage I–IV CRCs showed that STAT3 was significantly associated with poor outcomes and it supported the potential role of STAT3 in pro-tumor inflammatory transmission (24). Activation of STAT3 has been shown to drive downstream gene transcription, and its gene products subsequently promote tumor development and progression (25). Our study put forward for the first time that cynaropicrin can inhibit the proliferation of CRC and induce its apoptosis by targeting the LIFR/STATs axis *in vitro* and *in vivo* (Fig. 7), suggesting that cynaropicrin is a potential natural product with anti-tumor efficacy.

In recent years, LIFR has been found to play various roles in tumors. As an oncogene, LIFR contributes to the subsequent activation of STAT3 and AKT pathways in prostate cancer, inducing the expression of a series of proliferation-related and transfer genes (26, 27). In breast cancer, LIFR is a tumor suppressor and metastasis suppressor gene. Abnormal LIFR promoter methylation and down-regulation of protein expression occur, which induce migration, invasion, and metastatic colonization (28, 29). As described above, it is very important to identify the role of LIFR in target tumors. According to the existing studies and analyses, LIFR is highly expressed in CRC tumor tissues, which is related to the overall 5-year survival rate after surgery and plays the role of a carcinogen (30). Moreover, LIFR reflects the chemosensitivity of metastatic CRC to cetuximab, which may be used to predict the susceptibility of individuals to cetuximab chemotherapy (31, 32). Therefore, it is important to identify drugs that target LIFR in the treatment of CRC. Our results support the claim that cynaropicrin targeted LIFR to block downstream activation of the STAT protein; thereby blocking CRC progression. This suggests that cynaropicrin is a compound with therapeutic potential against CRC.

STAT signals are involved in immune function, cell growth, differentiation, hematopoietic and human cancer development, progression, metastasis, survival, and treatment resistance(33). Based on the results of the present studies, STAT3 may play a relatively important role in tumor and inflammatory biology, while STAT4 appears to be less involved (10). However, their role is due to transcriptional modifications controlled by the subtle and complex interactions of different STAT molecules. In the inflammatory pathways of fibroblasts, LIFR forms a molecular complex with STAT4, which initiates STAT4 activation. STAT4 then enters the nucleus and is recruited to the IL-6 promoter (34). A positive feedback loop

involving LIF, LIFR, and STAT4 drives sustained IL-6 transcription. In lamina propria lymphocytes of inflammatory bowel disease patients, LIF-activated STAT4 inhibits activation of the STAT3-dependent IL17a/IL17f promoter, while in intestinal epithelial cells, LIF bypasses abnormally low STAT4 levels and induces YAP gene expression by activating STAT3 (22). Therefore, our attempt to investigate the interaction between STAT3 and STAT4 proteins in CRC was meaningful. LIF stimulation can induce LIFR to recruit STAT3 and STAT4. Our experimental data demonstrated that cynaropicrin inhibited the formation of this complex and blocked the formation of STAT3-4 heterodimer. LIFR protein was positive when immunoprecipitation was performed using STAT3 primary antibody but negative with STAT4 primary antibody. We consider that it is probably a consequence of insufficient STAT4 protein abundance.

Cynaropicrin is one of the main active ingredients of artichoke. Maximum tolerated dose (MTD) was assessed in Swiss-Webster female rats for acute toxicity testing. The results showed that MTD for an intraperitoneal injection was 200 mg/kg and for oral administration was 400 mg/kg, which was much higher than the IC₅₀ value measured in CRC cells in our study (35). Therefore, the inference that cynaropicrin is safe and less toxic is basically reliable. In addition to its own anti-tumor efficacy, cynaropicrin increased the chemotherapeutic sensitivity of tumor cells, showing a mild to strong synergistic effect with cisplatin and docetaxel (19). As a natural product, it has obvious advantages if it can be an effective therapeutic drug. On the one hand, the compound is water-soluble and can therefore be configured with therapeutic injections to shorten the onset time and thus reduce the possible side effects (36). On the other hand, its simple structure and low cost of synthesis and manufacturing can reduce the medical treatment burden of patients (37).

Conclusions

In summary, our study suggested that cynaropicrin causes potential inhibition of CRC *in vitro* and *in vivo* by suppressing the activation of LIFR/STATs signaling pathway. Our findings, together with the work of our predecessors, reveal the potential therapeutic value of cynaropicrin. However, further strategies need to be elucidated to optimize the clinical use of cynaropicrin.

Abbreviations

BAX: BCL2-associated X protein; **BCL2**: B-cell lymphoma 2; **BSA**: Bovine serum albumin; **CRC**: Colorectal cancer; **DAPI**: 4',6-diamidino-2-phenylindole; **DMEM**: Dulbecco's Modified Eagle Medium; **DMSO**: Dimethyl sulfoxide; **FBS**: Fetal bovine serum; **GAPDH**: Glyceraldehyde 3-phosphate dehydrogenase; **H&E**: Hematoxylin-eosin staining; **IBD**: inflammatory bowel disease; **IC₅₀**: The half maximal inhibitory concentrations; **IL-6**: Interleukin 6; **JAK**: Janus Kinase; **LIF**: Leukemia inhibitory factor; **LIFR**: Leukemia inhibitory factor receptor; **MTD**: Maximum tolerated dose; **MTT**: Methylthiazolyldiphenyl-tetrazolium bromide; **OD**: Optical density; **PARP1**: Poly ADP-ribose polymerase 1; **PBS**: Phosphatebuffered saline; **PI**: Propidium Iodide; **PVDF**: Polyvinylidene Fluoride; **ROS**: reactive oxygen species; **RPMI**: Roswell Park Memorial Institute; **SEM**: Standard error of mean; **SD**: Standard deviation; **SDS-PAGE**: Sodium dodecyl sulfate-polyacrylamidegel; **SH2**: Src homology domain 2; **STATs**: Signal transducers and activators of

transcription; **STL**: Sesquiterpene lactone; **TBST**: Tris-Buffered Saline and Tween 20; **Trx**: Thioredoxin; **YAP**: Yes-associated protein.

Declarations

• Ethics approval and consent to participate

All animal studies were performed with an approved protocol by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

• Consent for publication

The authors declare that they agree to submit the article for publication.

• Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

• Competing interests

The authors declare that they have no competing interests.

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• Authors' contributions

DD Z, Y Z, and YQ X carried out the most experiments. XX D, WL H, B Z and ZG L analyzed the data and prepared the figure. HY Z prerevised the manuscript. CG Z, XY H and LX W conceived the idea and designed the research. CG Z, XY H and DD Z wrote the manuscript. All authors read and approved final version of the manuscript.

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Figures

Figure 1

Cynaropicrin inhibited CRC cell proliferation and colony formation. (A) The inhibition ratio was calculated by MTT assay in Hct116, RKO, DLD-1 cells. (B) Colony formation assay was performed using the above-mentioned three cells treated with or without Cynaropicrin. All potency values are expressed as mean $\pm$ SD of independent experiments in triplicate. Definition of statistical significance: $P < 0.05$.

Figure 2

Cynaropicrin-induced cell apoptosis in CRC cells. (A) Flow cytometric analysis and (B) Hoechst 33258 staining were performed to analyze the cell apoptotic rate after treatment with 0, 5, 7.5 and 10 mM Cynaropicrin for 24h in Hct116, RKO, and DLD-1 cells. (C) The expression levels of Cleaved PARP1, Bax and BCL2 in three cells were measured by Western blotting after incubation with Cynaropicrin at three

concentrations for 24h. All potency values are expressed as mean $\pm$ SD of independent experiments in triplicate. Definition of statistical significance: $P < 0.05$.

Figure 3

Cynaropicrin induced loss of migration potential in CRC cells. Transwell migration assay was performed in Hct116 and DLD1 cells.

Figure 4

Cynaropicrin inhibited the LIFR/STAT3 signaling pathway. (A)(B)DLD1 and RKO cells were treated with Cynaropicrin at different concentrations (0, 5, 7.5, and 10mM) or for different lengths of time (0, 2, 4, 8, 12, and 24h). Western blotting was used to detect the protein expression levels of LIF/LIFR, and P-STAT3/STAT3. RKO cells were pretreated for 24h and then stimulated with IL-6 (25 ng/mL) for 30 min. (C) Subcellular localization of STAT3 was detected by immunofluorescence staining and laser confocal

microscopy. (D) The expression of STAT3 protein in cytoplasm and nucleus was detected by Western blotting after extraction with a kit.

Figure 5

Cynaropicrin stimulation inhibited the formation of STAT3-STAT4 heterodimers. (A)(B) RKO cells were treated with (+) or without (-) Cynaropicrin for 24h, and then total cell protein was extracted to perform a co-immunoprecipitation assay using STAT3 antibody or STAT4 antibody. The immunoprecipitation complexes were further separated by Western blotting. (C) RKO cells were pretreated for 24h and then stimulated with IL-6 (25 ng/mL) for 30 min. Subcellular localization of STAT4 was detected by immunofluorescence staining and laser confocal microscopy.

Figure 6

Function of Cynaropicrin in tumor xenograft growth inhibition of CRC in vivo. (A) BALB/c mice were grouped into Control, Napabucasin 10mg/kg, Cynaropicrin 2.5 mg/kg, and Cynaropicrin 5 mg/kg. Tumor volumes were recorded every 2 days and the curve was finally plotted. (B) At the end of study mice were sacrificed, and the tumors were removed and weighed. (C) Gross view of xenograft tumor tissue (D) The expression levels of STAT3-related pathway proteins (LIFR, LIF, P-STAT3, and STAT3) and apoptotic proteins (Cl-PARP1, Bax, and Bcl2) in transplanted tumor tissues were detected by WB. (E) The mice were

weighed throughout the experiment. (F) No histological abnormalities were observed in H&E staining of heart, liver, lung and kidney in the four mice groups. All potency values are expressed as mean $\pm$ SD of independent experiments in triplicate. Definition of statistical significance: $P < 0.05$.

Figure 7

Schematic diagram of the antitumor mechanism of Cynaropicrin in CRC by blocking STATs activation into the nucleus by targeting LIFR